

**AMELIORATIVE EFFECT OF MELATONIN ON HISTOPATHOLOGICAL ALTERATIONS-INDUCED BY CARBON TETRACHLORIDE IN WISTAR RATS**

By

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## **Abstract**

Carbon tetrachloride (CCl<sub>4</sub>) is a well-known hepatotoxin and exposure to this volatile organic chemical, is known to induce oxidative stress and causes tissue damage, especially to the liver, kidney and central nervous system. In experimental animals CCl<sub>4</sub> has been shown to be carcinogenic. Melatonin (MEL), the principal hormone of pineal gland plays an important role in several biological processes. The present study was designed to evaluate the ameliorative effect of exogenous melatonin treatment on CCl<sub>4</sub>-induced oxidative stress and resultant hepatotoxicity in rats. Forty Wistar rats, weighing 240-260 g were divided into four (A, B, C and D) groups, each consisting of 10 rats. Group A rats served as control and received olive oil in quantities equivalent to the volume of MEL and CCl<sub>4</sub> administered subcutaneously. Group B and C rats received subcutaneous injections of CCl<sub>4</sub> solution (1 ml/kg body weight) in olive oil, twice in a week for 8 weeks. The rats in group C were additionally treated with MEL (5 mg/kg body weight, s.c.) for 3 days prior to administration of CCl<sub>4</sub>; and MEL treatment was continued for further 8 weeks at alternate days. Group D rats received MEL (5 mg/kg body weight, s.c.) treatment only for a period of 8 weeks at alternate days. Hepatic marker enzymes, alanine and aspartate aminotransferases (ALT and AST), also Ca<sup>2+</sup>, tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) were estimated in the sera of all studied groups of rat. Antioxidant status in the liver tissue was estimated by determining the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and glutathione-S-transferase (GST) as well as determination of thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) levels. For histopathological evaluation, liver of all rats were excised and processed for light microscopy. A significant increase in the activities of serum and homogenates ALT and AST were observed in CCl<sub>4</sub>-treated rats when compared with the control rats. Also, TBARS level increased significantly whereas GSH, SOD, CAT, GST levels were decreased in the liver homogenates of CCl<sub>4</sub>-treated rats. Melatonin treatment positively ameliorated the alterations in these biochemical variables in the CCl<sub>4</sub> + MEL-treated rats. Liver sections of CCl<sub>4</sub>-treated group showed, marked necrosis, inflammatory cells infiltration, fatty degeneration and hydropic degeneration of hepatocytes. In contrast, these deleterious histopathological alterations resulting from CCl<sub>4</sub> hepatotoxin were absent after MEL treatment in CCl<sub>4</sub> + MEL group of rats. Our results demonstrated that melatonin exerts ameliorative effect on the liver by restoring the activities of hepatic marker enzymes and reversing the distorted micro-anatomical changes induced by CCl<sub>4</sub> via enhancement of antioxidative defense activities, decreased expression of procytokines and restoration of Ca<sup>2+</sup> homeostasis.

## **Key Words:**

Carbon tetrachloride; Melatonin; Hepatotoxicity; Hepatic marker enzymes; Antioxidants

**INTRODUCTION**

Carbon tetrachloride (CCl<sub>4</sub>) does not occur naturally, it is a clear liquid with sweet smell that can be detected at low levels (Doherty, 2000). Exposure to various compounds including a number of environmental pollutants and drugs can cause cellular damages through metabolic activation of those compounds to a highly reactive oxygen species (ROS). Free radical induced lipid peroxidation is believed to be one of the major causes of cell membrane damage leading to a number of pathological situations (Slater, 1984). CCl<sub>4</sub> was formerly used for metal degreasing and as dry-cleaning, fabric-spotting, and fire extinguisher fluids, grain fumigant and reaction medium. Because of its harmful effects, these uses are now banned and it is only used in some industrial applications (DeShon, 1979). The primary routes of potential human exposure to CCl<sub>4</sub> are inhalation, ingestion, and dermal contact. High exposure to CCl<sub>4</sub> can cause liver, kidney and central nervous system damage, and liver is especially sensitive to CCl<sub>4</sub> because of its role as the body's principal site of metabolism (Sakata *et al.*, 1987). Liver fibrosis is the common end-stage of most chronic liver disease, regardless of etiology, and its progression leads to cirrhosis and liver cancer. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, but the major risk factors include hepatitis B and C viral infections, exposure to dichloromethane compounds and aflatoxin B<sub>1</sub> and alcoholic liver disease (Hecht, 1997). A number of endogenous and exogenous cancer risk factors generate oxygen free radicals *in vivo*. Therefore the role of oxygen-derived free radicals and lipid peroxidation has attracted considerable attention (Gebhardt, 2002; Das *et al.*, 2005). Volatile organic compounds such as CCl<sub>4</sub> are a class of solvents to which many people are exposed occupationally and environmentally. Early studies of dichloromethane, CCl<sub>4</sub> and 1,1-dichloroethylene revealed susceptibility of rats to liver damage by these chemicals (Bruckner *et al.*, 1984). It has been found that metabolism of CCl<sub>4</sub> involves in the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum (Slater and Sawyer, 1971). Therefore, it has become a task to prevent and cure hepatic damage by eliminating free radicals and prevent lipid peroxidation through a terminal antioxidant like melatonin (Esrefoglu *et al.*, 2005).

Hepatic fibrosis represents the generalized response of the liver to injury and is characterized by excessive deposition of extracellular matrix. The cellular basis of this process is complex and involves interplay of many factors, of which cytokines are prominent (Zengdun *et al.*, 1997). The result of injury to the liver, regardless of type (i.e., virus, toxin, or biliary obstruction), is fibrosis. During the fibrogenic response to injury, stellate cells (also known as lipocytes, Ito, or perisinusoidal cells) differentiate into matrix-producing cells, which in large part are responsible for hepatic fibrosis (Friedman, 1993). A number of cytokines have been implicated in the pathogenesis of hepatic fibrosis via direct or indirect effects on stellate cells and transforming growth factor  $\beta$  (TGF- $\beta$ ), tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ) and interferon  $\gamma$  (IFN- $\gamma$ ) (Czaja *et al.*, 1989). Potential sources of cytokines in the hepatic wounding response include macrophages (hepatic Kupffer cells), hepatocytes, stellate cells, and natural killer (NK) cells. In addition, lymphocytes, including CD4<sup>+</sup> T helper (Th) cells reside in the liver (Tiegs *et al.*, 1992) and represent a further potential source of cytokines. Cytokines are multipotent low molecular weight proteins which, in addition to

the important functions in the immune system, also exert various actions in the liver. IL-1 is one of the most studied pro-inflammatory cytokines, TNF- $\alpha$  is produced predominantly by macrophages and lymphocytes (Jones *et al.*, 1989; Jones *et al.*, 1994), and it acts on fibroblasts and endothelial cells, not only in producing inflammatory reactions and fibrosis but also eliciting the production of other inflammatory cytokines and adhesion molecules (Hatzistilianou *et al.*, 1997; Lantz *et al.*, 1991).

Mammalian cells are equipped with both enzymic and non-enzymic antioxidants defenses with different efficacies that protect animals against oxidative abuse caused by wide range of hepatotoxicants including CCl<sub>4</sub> (Karbownik *et al.*, 2001). Melatonin, N-acetyl-5-methoxytryptamine is a hormone found in all living creatures from algae (Caniato *et al.*, 2003) to humans, at levels that vary in a diurnal cycle. In higher animals MEL is produced by pinealocytes in the pineal gland. Melatonin participates in many important physiological functions, including anti-inflammatory (Cuzzocrea and Reiter, 2002), also, it is a powerful antioxidant that can easily cross cell membranes and the blood-brain barrier (Hardeland, 2005). Unlike other antioxidants, MEL does not undergo redox cycling, once oxidized; it cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals (Tan *et al.*, 2000). Redox cycling may allow other antioxidants such as vitamin C to act as pro-oxidants, counterintuitively promoting free radical formation. Furthermore, MEL has been demonstrated to prevent damage to DNA by some carcinogens, stopping the mechanism by which they cause cancer (Karbownik *et al.*, 2001; Carrillo-Vico *et al.*, 2005). Melatonin is an immunomodulatory agent that enhances T-cell production and when taken in conjunction with calcium, it is a potent immunostimulator of the T-cell response (Vijayalaxmi *et al.*, 2002). It holds the unique position of being the only known chronobiotic regulator of neoplastic cell growth. Both *in vitro* and *in vivo*, MEL has been found to inhibit neoplastic growth and to delay tumor progression (Tan *et al.*, 1993). In addition, MEL protects liver injury induced by endotoxin shock and ischemia/reperfusion in rats through its antioxidant action (Sener *et al.*, 2003).

Beneficial effects of exogenous MEL on vital organs like brain, liver, kidney and lungs as well as cardiovascular system have been largely reviewed (Zenebe *et al.*, 2001). Therefore, the present study was designed to (i) evaluate ameliorative effect of exogenous melatonin on chronic liver damage; fibrosis and necrosis induced by CCl<sub>4</sub> in rats, and (ii) analyze degree of liver injury via antioxidative and anti-inflammatory mechanisms.

## **MATERIALS AND METHODS**

### **Animal care and monitoring**

This study was carried out in healthy, male and female Wistar rats, weighing 240-260 g. The animals were housed under standard laboratory conditions of light, temperature (21 $\pm$ 2°C) and relative humidity (55 $\pm$ 5%). The animals were given standard rat pellets and tap water *ad libitum*. The rats were randomly divided into four experimental groups: A (control), B (CCl<sub>4</sub>), C (CCl<sub>4</sub> + MEL) and D (MEL), each group consisted of 10 rats. The experimental protocol and procedures used in this study were approved by the Ethics

Committee of the University of KwaZulu-Natal, Durban 4000, South Africa; and conform with the “*Guide to the Care and Use of Animals in Research and Teaching*” [Published by the Ethics Committee of the University of Durban-Westville, Durban 4000, South Africa].

### **Experimental design**

Animals were divided into four groups of ten rats each. Group A rats were treated with olive oil in quantities equivalent to the volume of CCl<sub>4</sub> and MEL administered subcutaneously. The rats in groups B and C were treated with CCl<sub>4</sub> (1 ml/kg body weight, s.c.) in a 1:1 solution with olive oil. The rats in group C were additionally treated with MEL (5 mg/kg/, s.c.) for 3 days prior to administration of CCl<sub>4</sub>; and MEL treatment was continued for further 8 weeks at alternate days. Melatonin was dissolved in (9 g/L) saline with absolute ethanol (<math>\leq 0.1\text{ ml/L}</math>) and stored at -20°C until used. Group D rats received MEL (5 mg/kg/, s.c.) treatment for a period of 8 weeks, at alternate days. All the animals were fasted for 16 hours, but still allowed free access to water, before the commencement of our experiments. At the end of experimental period of 8 weeks, the animals were sacrificed by cervical dislocation; serum/plasma samples and liver tissue were collected and stored at -80°C for analysis.

### **Liver histopathology**

Liver tissues were excised from sacrificed animals, weighed, and thin liver slices were cut, fixed in 4% paraformaldehyde and were sequentially embedded in paraffin wax blocks. Five  $\mu\text{m}$  thick sections were cut, and stained with hematoxylin-eosin (HE) and masson’s trichrome for conventional morphological evaluation, then examined under light microscope (Olympus). The degree of hepatic necrosis and fibrosis were determined by a semi-quantitative method (Pilette *et al.*, 1998).

### **Liver glycogen assay**

Total hepatic glycogen was estimated as marker of hepatic energy status. Glycogen level in the hepatic extract was determined as described by Jaime *et al.* (1997). Liver was removed immediately after euthanization, rinsed with cold saline, weighed and then isolate a piece of tissue, frozen in liquid nitrogen. Hepatic tissue extract (0.1 g) was dissolved in 30% KOH and heated at 100°C for 10 min, followed by 3-min room temperature incubation. The sample was diluted (1:10) with KOH and vortexed. Anhydrous ethanol (1.1-1.2 volumes) was added to precipitate the glycogen from alkaline digestate, and samples were centrifuged at 5,700 rpm for 15 min. The supernatant was carefully removed, and the pellet was resuspended in 0.5 ml distilled H<sub>2</sub>O. One milliliter of 0.2% anthrone reagent (0.2 g in 100 ml of 98% H<sub>2</sub>SO<sub>4</sub>) was added rapidly and mixed, and the mixture was incubated at room temperature for 30 min. The samples were then measured at 620 nm with a spectrophotometer.

### **Serum and homogenate alanine and aspartate aminotransferases (ALT and AST)**

The activities of ALT and AST were measured as biomarkers of liver injury. Whole blood was centrifuged at 4 700 rpm for 10 min at 4°C and ALT, AST and total protein were determined spectrophotometrically with an automatic analyzer (Cobas Mira; Roche, Rotkreuz, Switzerland) using commercially available kits (Roche Diagnostics). Their activities were expressed as an international unit (IU/L).

ALT and AST activities in liver homogenates were also determined using a quantitative, colorimetric end-point assay kit (Procedure No. 104, Sigma Diagnostics, St. Louis, MO) that used  $\alpha$ -ketoglutaric acid as the substrate and that detected production of pyruvic acid. Results were expressed as units/mg protein.

### **Preparation of tissue homogenate**

The harvested liver tissues were rinsed in cold physiological saline, cleaned of gross adventitial tissue, blotted dry and stored in a Biofreezer at  $-80^{\circ}\text{C}$  until analyzed. Liver tissue was homogenized with Potter Elvehjem homogenizer. 10% homogenates were prepared in 6.7 mM phosphate buffer, pH 7.4 and centrifuged at 10,000 rpm for 10 min at 4°C, and the resultant supernatant was used for measurement of antioxidant enzymes. For the determination of lipid peroxidation, liver tissue was homogenized in 1.15% KCl solution to obtain a 10% (w/v) homogenate. Protein content of liver homogenates was determined by using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Company, Rockford, IL). All enzyme activities were expressed as units/mg protein.

### **Serum calcium concentration and subcellular fraction levels**

A serum volume of 100  $\mu\text{l}$  was diluted to 5 ml, and potassium was added to give a final concentration of 5000  $\mu\text{g/ml}$  in order to suppress ionization. The sample was acidified with concentrated HCl. Atomic absorption spectrophotometer (Varian AA-875) equipped with an adjustable uptake nebulizer was used. Calcium determination was carried out at 422.7 nm line.

Subcellular fraction of homogenized liver in a buffer containing 0.25 mol/l sucrose and 10 mmol/l HEPES/KOH (pH 7.4), was obtained as describe by Diaz-Munoz *et al.* (1996). Total  $\text{Ca}^{2+}$  measurement was carried out, thus, both mitochondrial and cytosolic fractions were deproteinized by adding Perchloric acid chloric (IV) acid ( $\text{HClO}_4$ ) (6% w/v final concentration) and centrifuged in the presence of Lanthanum chloride ( $\text{LaCl}_3$ ) and kept frozen ( $-80^{\circ}\text{C}$ ) until use.  $\text{Ca}^{2+}$  content from the deproteinized fractions was measured by atomic absorption spectrophotometer at 422.7 nm (Varian AA-875).

### **Serum tumor necrosis factor-alpha (TNF- $\alpha$ ) and interkeukin-1 (IL-1)**

Blood samples were collected and centrifuged at 3000 rpm for 15 min at 4°C. Serum aliquots were frozen and stored at  $-80^{\circ}\text{C}$  until the assays for serum TNF- $\alpha$  and IL-1 was performed. The specimens were thawed immediately before analysis and used at volumes indicated by the manufacturers. The analyses were performed with 96-well microliter plate Enzyme-linked immunosorbent assay (ELISA) kits (DPC Diagnostic Products

Corporation, Los Angeles, CA), an automatized random access immunoassay system developed for the measurement of immune parameters in single samples (Babson, 1991). The serum was assayed strictly according to procedures described by the instruction of the manufacturer's protocol. Test sensitivity was 6.5 pg/ml for IL-1 and 5 pg/ml for TNF- $\alpha$ .

### **Biochemical assays:**

#### **Superoxide Dismutase Activity (SOD)**

Liver SOD activity was assayed by the method of Kakkar *et al.*, (1984). Reaction mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 mM, pH 7.0), 0.1 ml of phenazine methosulphate (PMS) (186  $\mu$ M), 0.3 ml of nitro blue tetrazolium (NBT) (300  $\mu$ M). 0.2 ml of the supernatant obtained after centrifugation (1500 x g, 10 min followed by 10,000 x g, 15 min) of 10% liver homogenate was added to reaction mixture. Enzyme reaction was initiated by adding 0.2 ml of NADH (780  $\mu$ M) and stopped precisely after 1 min by adding 1 ml of glacial acetic acid. Amount of chromogen formed was measured by recording color intensity at 560 nm. Results are expressed as units/mg protein.

#### **Glutathione Peroxidase Activity (GSH-Px)**

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide (Tappel, 1978). 100  $\mu$ L of enzyme sample was incubated for five minutes with 1.55 ml stock solution (prepared in 50 mM Tris buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50  $\mu$ L of cumene hydroperoxide (1 mg/ml), and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1  $\mu$ mol of NADPH to NADP per minute. Results are expressed as units/mg protein.

#### **Catalase Activity (CAT)**

The activity of CAT was measured using its peroxidatic function according to the method of Johansson and Borg, (1988). 50  $\mu$ L potassium phosphate buffer (250 mM, pH 7.0) was incubated with 50  $\mu$ L methanol and 10  $\mu$ L hydrogen peroxide (0.27%). The reaction was initiated by addition of 100  $\mu$ L of enzyme sample with continuous shaking at room temperature (20°C). After 20 minutes, reaction was terminated by addition of 50  $\mu$ L of 7.8 M potassium hydroxide. 100  $\mu$ L of purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole, 34.2 mM in 480 mM HCl) was immediately added, and the mixture was again incubated for 10 minutes at 20°C with continuous shaking. Potassium periodate (50  $\mu$ L 65.2 mM) was added to obtain a colored compound. The absorbance was read at 550 nm in a spectrophotometer. Results are expressed as micromoles of formaldehyde produced/mg protein.

### **Reduced glutathione (GSH)**

GSH level was measured following the method of Ellman (1959), modified by Hissin and Hilf (1973) The homogenate (720  $\mu$ l) was double diluted and 5% TCA was added to it to precipitate the protein content of the homogenate. After centrifugation (10,000 x g for 5 minutes) the supernatant was taken, 5,5'-dithiolbis-2-nitrobenzoic acid (DTNB) solution (Ellman's reagent) was added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentrations of standard GSH solution (1 mg/ml). With the help of the standard graph, GSH contents in the liver homogenates of the experimental animals were calculated.

### **Glutathione-S-transferase (GST)**

GST catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. GST activity was measured by the method of Habig and Jakoby (1974). The reaction mixture contained suitable amount of the enzyme (25  $\mu$ g of protein in homogenates), 1 ml of  $\text{KH}_2\text{PO}_4$  buffer, 0.2 ml of EDTA, 0.1 ml of 1-chloro-2,4-dinitrobenzene (CDNB), and GSH. The reaction was carried out at 37°C and monitored spectrophotometrically by the increase in absorbance of the conjugate of GSH and CDNB at 340 nm. A blank was run in absence of the enzyme. One unit of GST activity is 1  $\mu$ mol product formation per minute.

### **Thiobarbituric Acid Reactive Substances (TBARS)**

The product of the reaction between malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were measured by a modified method of Esterbauer and Cheesman, (1990). For each sample to be assayed, four tubes were set up containing 100, 150, 200 and 250  $\mu$ L of tissue homogenate, 100  $\mu$ L of 8.1% SDS, 750  $\mu$ L of 20% acetic acid, and 750  $\mu$ L of 0.8% aqueous solution of TBA. The volume was made up to 4 ml with distilled water, mixed thoroughly and heated at 95°C for 60 minutes. After cooling, 4 ml of n-butanol was added to each tube, the contents mixed thoroughly, and then centrifuged at 3000 rpm for 10 minutes. The absorption of the clear upper (n-butanol) layer was measured using a Shimadzu UV-1601 (Japan) spectrophotometer at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex  $1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$  and was expressed in  $\mu$ mol TBARS/mg tissue protein.

### **Statistical Analysis**

The data obtained were expressed as means ( $\pm$ SEM), and analyzed using repeated measures of variance. The differences between the means were analyzed statistically with one-way analysis of variance (ANOVA; 95% confidence interval). Values of  $p < 0.05$  were taken to imply statistical significance.

## RESULTS

### Body and liver weights

Table 1 shows the changes in body and liver weights of all experimental animal groups. The body weight of CCl<sub>4</sub>-treated group rats significantly decreased when compared with the control and MEL-treated groups of rat. The CCl<sub>4</sub> + MEL group insignificantly showed low body weights. The liver weight of CCl<sub>4</sub>-treated group B rats significantly increased ( $p < 0.05$ ) when compared with the groups A, C and D rats (Table 1).

**Table 1.** Changes in body and liver weights of control, CCl<sub>4</sub>-, CCl<sub>4</sub>- + MEL-, and MEL-treated rats.

Parameters	Control	CCl <sub>4</sub>	CCl <sub>4</sub> + MEL	MEL
Body weights (g)	252.7±9.3	227.8±5.4 <sup>a</sup>	238.3±7.6 <sup>a</sup>	251.6±1.8 <sup>a</sup>
Liver weights (g)	9.82±1.5	13.27±1.3 <sup>b</sup>	10.56±1.4 <sup>b</sup>	9.88±1.8 <sup>b</sup>

Values are expressed as means (±SEM) of 8 rats for all groups. <sup>a,b</sup> Significant difference ( $p < 0.05$ ) in the same row between various treatments and control group A rats.

### Histopathological findings

In normal group A rats, there was no pathological abnormalities. Liver parenchyma showed normal morphology and hepatocytes were arranged around the central vein. No congestion and inflammatory cell infiltrations in and around centrilobular area (Fig. 4A). CCl<sub>4</sub> treatment induced severe pathological abnormality. Hepatocytes were with marked vacuolization, necrotic swollen hepatocytes with granular cytoplasmic characteristics representative of increased amount of endoplasmic reticulum. Inflammatory cell infiltrations with mild fibrosis were evident around the bridging necrotic tissue. Congestion in liver sinusoids was significant with scattered infiltration of inflammatory cells (Fig. 4B). In CCl<sub>4</sub> + MEL-treated group, the liver section showed attenuated necrosis and reduced immigration inflammatory cells. Liver parenchyma was well preserved with radially arranged hepatocytes around the central vein. Regular sinusoidal structures were seen without congestion (Fig. 4C). MEL-treated group of rats showed normal lobular architecture with central veins and radiating hepatic cords (Fig. 4D).

### Liver glycogen contents

Carbon tetrachloride liver injury results in breakdown of glycogen, causing significant reduction in hepatic glycogen stores. Liver glycogen levels significantly decreased ( $p < 0.05$ ) in the group B rats treated with CCl<sub>4</sub> when compared with the control group of rats. Glycogen levels became markedly reduced in these rats, especially at longstanding hepatotoxic states. Glycogen values for the control, CCl<sub>4</sub>, CCl<sub>4</sub> + MEL and MEL-treated rats were found to be 1.97±1.32, 0.35±1.26, 1.18±1.35 and 1.92±1.16 mg/g tissue, respectively (Fig 1A).

**Serum and liver homogenate ALT and AST activities**

The activities of serum alanine and aspartate aminotransferases significantly increased ( $p < 0.05$ ) in CCl<sub>4</sub>-treated rats when compared with the control- and MEL-treated groups of rat but insignificantly rise ( $p < 0.01$ ) in CCl<sub>4</sub> + MEL rats (Table 2). Enzyme activities are expressed as I.U. ( $\mu\text{mol}/\text{min}/\text{l}$  of serum).

As shown in Table 2, CCl<sub>4</sub> significantly increased ( $p < 0.05$ ) ALT and AST activities in liver homogenates when compared with the control. MEL treatment equally reduced the activities of ALT and AST in the MEL + CCl<sub>4</sub> as well as MEL groups of rats (Table 2).

**Table 2.** Changes in serum and liver homogenates alanine and aspartate aminotransferase (ALT and AST) activities

	Control	CCl <sub>4</sub>	CCl <sub>4</sub> + MEL	MEL
ALT (IU/L)	42.56±2.34	132.32±1.56 <sup>a</sup>	56.26±5.42 <sup>b</sup>	38.28±1.42
AST (IU/L)	98.16±4.19	248.23±8.40 <sup>a</sup>	122.21±5.30 <sup>b</sup>	92.25±6.20
ALT (U/mg protein)	35.46±2.23	58.61±3.13 <sup>a</sup>	39.55±1.75 <sup>b</sup>	31.24±3.15
AST (U/mg protein)	24.34±1.54	36.81±2.64 <sup>a</sup>	27.70±4.12 <sup>b</sup>	25.18±3.28

Values are expressed as means ( $\pm$ SEM) of 8 rats. <sup>a</sup>Significant difference ( $p > 0.05$ ) between CCl<sub>4</sub>-treated groups and control. <sup>b</sup>Significant difference ( $p < 0.05$ ) between CCl<sub>4</sub>+MEL-treated and control groups.

**Table 3.** Changes in the activities of SOD, GSH-Px, CAT, GST, and levels of GSH and TBARS in liver homogenates of experimental animal groups

	Control	CCl <sub>4</sub>	CCl <sub>4</sub> + MEL	MEL
SOD (U/mg protein)	44.12±0.14	29.30±0.52 <sup>a</sup>	36.24±0.62	48.18±0.40
GSH-Px ( $\mu\text{mol}/\text{mg}$ protein)	19.22±0.29	12.34±0.36 <sup>a</sup>	16.35±0.12	20.40±0.84
CAT ( $\mu\text{mol}/\text{mg}$ protein)	56.53±0.62	24.28±0.71 <sup>a</sup>	49.76±0.26	58.80±0.38
GST ( $\mu\text{mol}/\text{mg}$ protein)	8.28±0.42	4.86±0.92 <sup>a</sup>	6.94±0.56	8.66±0.36
GSH ( $\mu\text{g}/\text{mg}$ protein)	16.14±0.87	9.64±0.56 <sup>a</sup>	14.32±0.76	17.24±0.96
TBARS (nmol/mg protein)	92.26±0.85	148.12±1.24 <sup>b</sup>	98.72±1.64	88.46±1.58

Values are expressed as means ( $\pm$ SEM) of 8 rats. <sup>a</sup>Significantly decreased ( $p > 0.05$ ) between CCl<sub>4</sub>-treated groups and control. <sup>b</sup>Significantly increased ( $p < 0.05$ ) between CCl<sub>4</sub>-treated and control groups.

**Table 4.** Effects of MEL on serum TNF- $\alpha$ , IL-1 level, serum and subcellular Ca<sup>2+</sup> (nmol Ca<sup>2+</sup>/mg protein) concentrations in rats with CCl<sub>4</sub>-induced hepatic injury

	Control	CCl <sub>4</sub>	CCl <sub>4</sub> + MEL	MEL
TNF- $\alpha$ (pg/ml)	5.36 $\pm$ 1.74	13.92 $\pm$ 1.43 <sup>a</sup>	6.16 $\pm$ 1.08 <sup>b</sup>	5.18 $\pm$ 1.12
IL-1 (pg/ml)	153.16 $\pm$ 9.87	312.84 $\pm$ 7.56 <sup>a</sup>	178.64 $\pm$ 9.85 <sup>b</sup>	148.25 $\pm$ 7.94
Serum Ca <sup>2+</sup>	8.86 $\pm$ 0.13	58.51 $\pm$ 1.33 <sup>a</sup>	14.25 $\pm$ 1.45 <sup>b</sup>	7.28 $\pm$ 1.75
Cytosol Ca <sup>2+</sup>	0.52 $\pm$ 0.04	0.78 $\pm$ 0.06 <sup>a</sup>	0.63 $\pm$ 0.09 <sup>b</sup>	0.49 $\pm$ 0.05
Mitochondria Ca <sup>2+</sup>	1.90 $\pm$ 0.25	5.75 $\pm$ 0.71 <sup>a</sup>	2.24 $\pm$ 0.38 <sup>b</sup>	1.89 $\pm$ 0.29

Values are expressed as means ( $\pm$ SEM) of 8 rats. <sup>a</sup>Significant difference ( $p > 0.05$ ) between CCl<sub>4</sub>-treated groups and control. <sup>b</sup>Significant difference ( $p < 0.05$ ) between CCl<sub>4</sub>+MEL-treated and MEL groups.

### Serum and subcellular Ca<sup>2+</sup> concentrations in rats with CCl<sub>4</sub>-induced hepatic injury

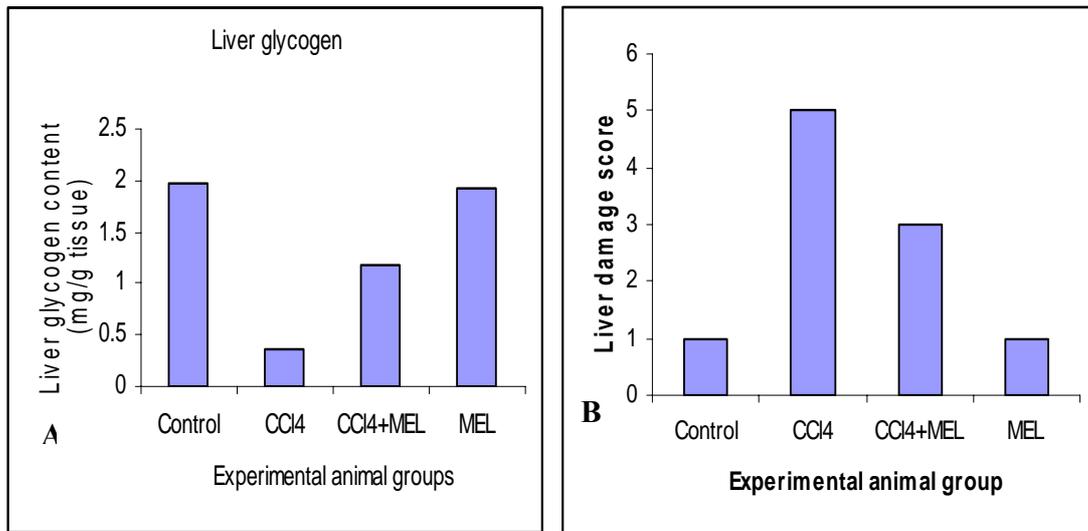
As shown in Table 4, CCl<sub>4</sub> significantly increased ( $p < 0.05$ ) Ca<sup>2+</sup> concentrations of both serum and subcellular fractions of liver homogenates when compared with the control group. Administration of MEL significantly decreased ( $p < 0.05$ ) Ca<sup>2+</sup> levels in the group C rats whereas it has no effects on group D rats (Fig. 2A, 2B and 2C).

### Serum tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-1 (IL-1)

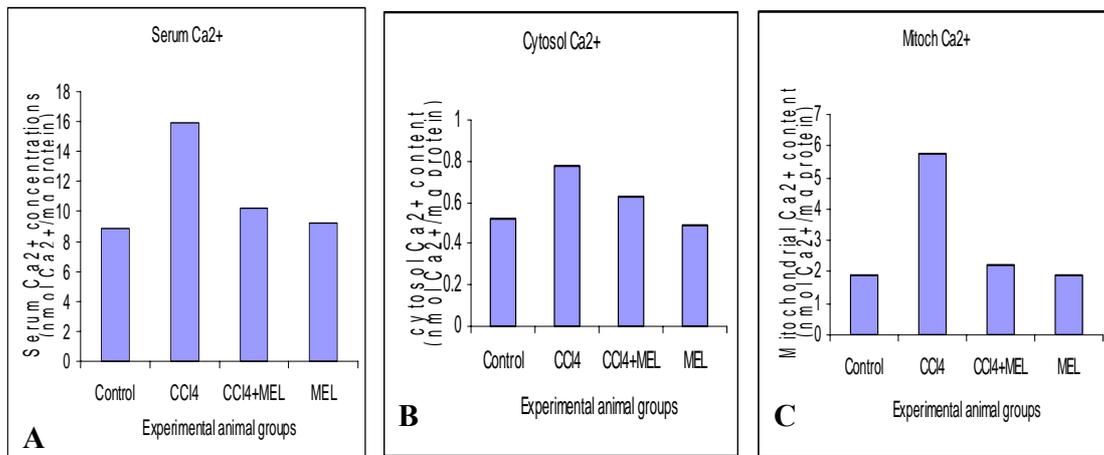
Table 4 shows the summary of serum TNF- $\alpha$  and IL-1 in all the experimental animal groups. Levels of TNF- $\alpha$  and IL-1 were significantly elevated ( $p < 0.05$ ) in CCl<sub>4</sub>-treated rats when compared with the control group. Melatonin treatment significantly ( $p < 0.05$ ) inhibited the production of the two indices levels in CCl<sub>4</sub>+ MEL-treated rats but has no effect in group D rats (Fig. 3A and 3B).

### Biochemical findings

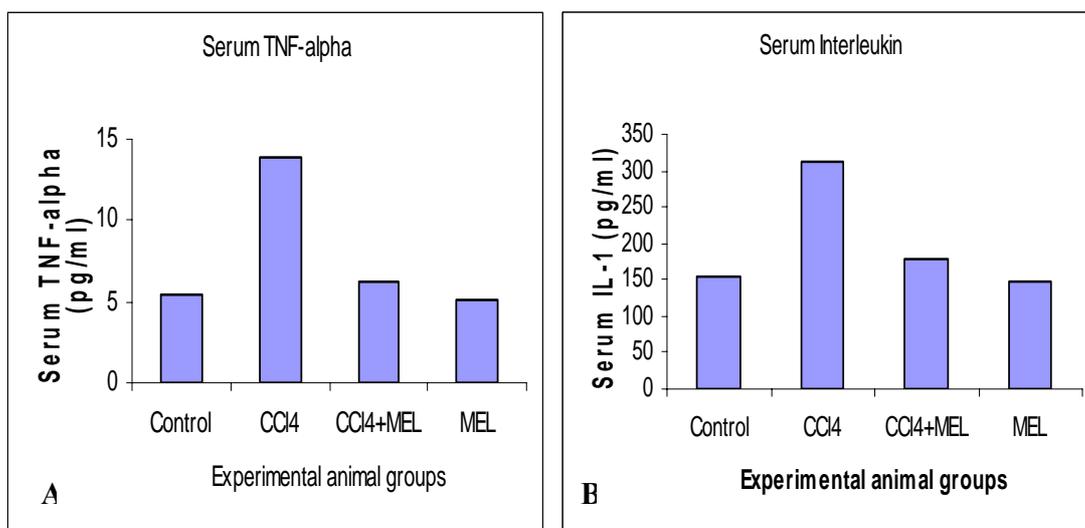
Table 3 shows the effects of melatonin on biochemical variables in the tissue homogenates of all experimental animal groups suggestive of oxidative stress in CCl<sub>4</sub>-treated rats. There was clear evidence that CCl<sub>4</sub>-induced hepatic injury was associated with free radical injury and oxidative stress. Oxidative stress was characterized by increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. Effects of CCl<sub>4</sub> and CCl<sub>4</sub> + MEL treatments on hepatic tissue's SOD, GSH-Px, CAT, GST, GSH and TBARS are presented in Table 3. The hepatic antioxidant activity of SOD, GSH-Px, CAT, GST and GSH significantly decreased ( $p < 0.05$ ), while hepatic TBARS significantly increased in the CCl<sub>4</sub>-treated, group of rats. The control group of rats maintained optimal value activity of the antioxidants studied. Administration of melatonin significantly ( $p < 0.05$ ) decreased the elevated TBARS, and also significantly increased ( $p < 0.05$ ) the reduced antioxidant enzyme activities. Furthermore, MEL proved significantly better in restoring the altered activity of antioxidant enzymes like SOD, GSH-Px, CAT, GST, GSH and TBARS towards their normal values in the liver homogenates. The animals treated with melatonin alone showed no significant change in the levels of GSH and in the activities of GST related enzymes (Table 3).



**Fig. 1.** Illustrates (A) chemical quantification of glycogen levels in the liver (B) quantification of liver damage score (scale 1-5), carried out under double-blind conditions in rats with hepatic injury after CCl<sub>4</sub> and MEL treatments. Results are expressed as means of 8 observations.



**Fig. 2.** Illustrates (A) Change of serum Ca<sup>2+</sup> concentrations, (B) Ca<sup>2+</sup> cytosol and (C) Mitochondrial Ca<sup>2+</sup> contents on rats with hepatic injury after CCl<sub>4</sub> and MEL treatments. Results are expressed as means of 8 observations.



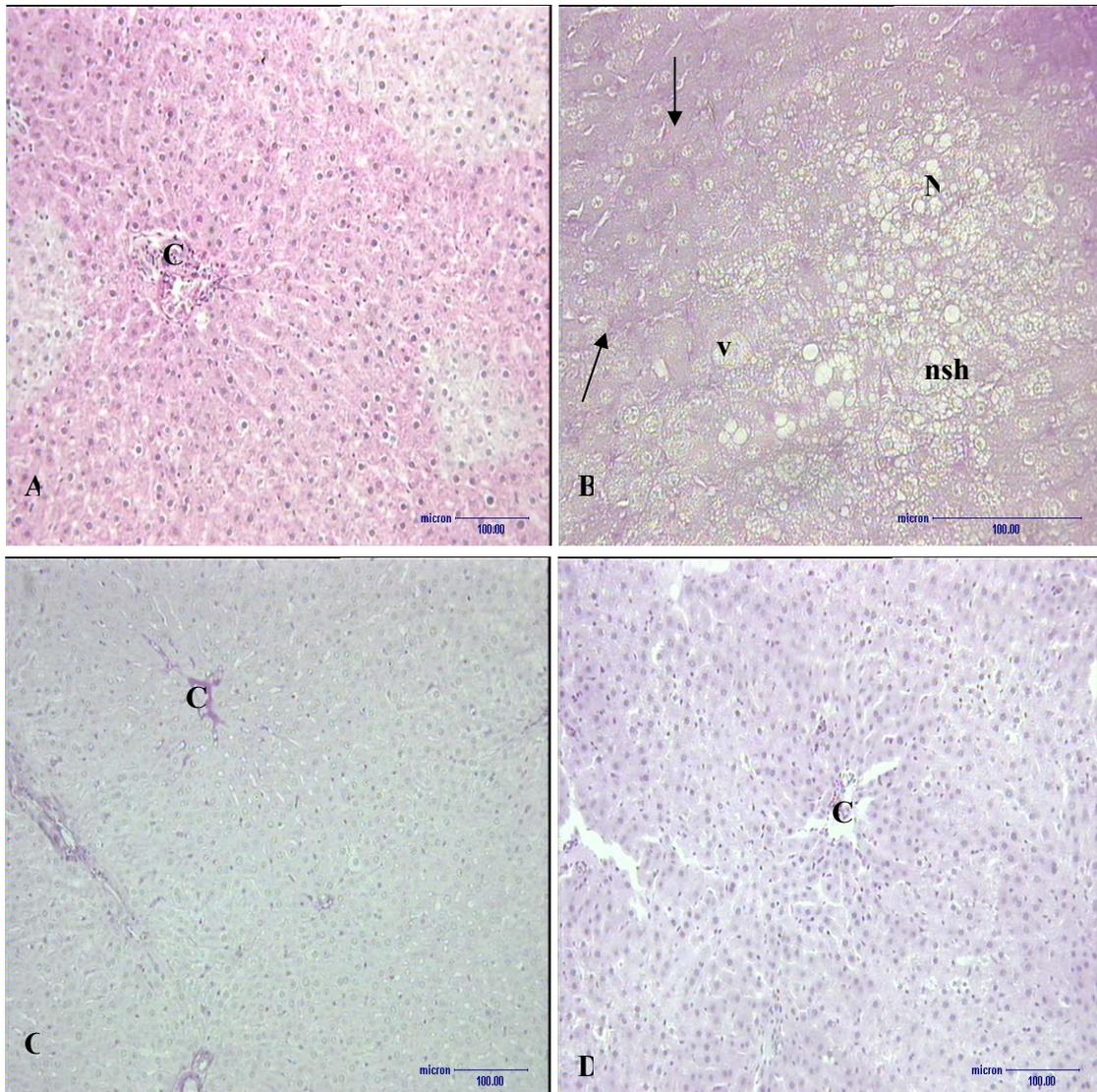
**Fig. 3.** Illustrates (A) serum tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels and (B) serum interleukin-1 levels in rats with hepatic injury after CCl<sub>4</sub> and MEL treatments. Results are expressed as means of 8 observations.

## DISCUSSION

Liver fibrosis is a common pathological process of hepatic disease, leading to the development of irreversible cirrhosis in humans and experimental animals. If treated properly at fibrosis stage, cirrhosis could be prevented (Riley and Bhatti, 2001). Liver fibrosis is characterized by increased deposition and altered composition of extracellular matrix, such that there is an excess of collagens (Friedman, 1993). When advanced, the liver architecture is distorted by dense bands of collagens that link vascular structures and surround islands of regenerating parenchymal cells; these changes are characteristic of cirrhosis. On this basis, administration of MEL *in vivo* resulted in marked reduction of liver injury, as demonstrated by significant reduction of serum aminotransferase concentration and amelioration of severe hepatic pathological abnormalities. Meanwhile, MEL decreased MDA content and increased SOD activity in liver homogenates. Furthermore, MEL significantly reduced liver glycogen content, serum Ca<sup>2+</sup>, TNF- $\alpha$  and IL-1 levels. Based on the current results, we propose that the mode of melatonin's hepatic protective action against CCl<sub>4</sub>-induced liver injury is, at least in part, related to its immunoregulatory, antioxidative and anti-inflammatory properties.

A number of chemicals including various environmental toxicants and clinically useful drugs can cause severe cellular damages in different organs of the body through metabolic activation to highly reactive substances such as free radicals. Carbon tetrachloride (CCl<sub>4</sub>) is one of such widely used environmental toxicant to experimentally induce animal models of acute hepatic damage. CCl<sub>4</sub> is metabolized by cytochrome P450 2E1 to trichloromethyl radical (CCl<sub>3</sub><sup>•</sup>). CCl<sub>3</sub><sup>•</sup> and its highly reactive derivative, the trichloromethylperoxyl radical (Cl<sub>3</sub>COO<sup>•</sup>), are assumed to initiate free radical-mediated

lipid peroxidation leading to accumulation of lipid peroxidation products that cause hepatic injury (Aleynik *et al.*, 1997).



**Fig. 4.** Photomicrographs of liver sections stained with masson's trichrome under light microscope. (A) Control rats showing normal liver parenchyma and hepatocytes were arranged around the central vein (C). No evidence of congestion or inflammation was observed in the sinusoid. (B)  $\text{CCl}_4$ -treated rats showing marked vacuolization (v), necrotic swollen hepatocytes (nsh) with granular cytoplasmic characteristics representative of increased amount of endoplasmic reticulum and inflammatory cells arranged around the necrotic (N) tissue. (C)  $\text{CCl}_4$  + MEL-treated rats showing attenuated necrosis, reduced inflammatory cells and well preserved liver parenchyma with radially arranged hepatocytes around the central vein. (D) MEL-treated rats showing normal lobular architecture with central veins and radiating hepatic cords. Bar = 100 $\mu\text{m}$ .

These radicals are capable of initiating a chain of lipid peroxidation reactions by abstracting hydrogen from polyunsaturated fatty acids (PUFA). Peroxidation of lipids, particularly those containing PUFA, can dramatically change the properties of biological membranes, resulting in severe cell damage and play a significant role in pathogenesis of diseases. This phenomenon results in the generation of ROS, (like superoxide anion  $O_2^-$ ,  $H_2O_2$  and hydroxyl radical  $OH^\cdot$ ). Evidence suggests that various enzymatic and non-enzymatic systems have been developed by mammalian cells to cope with ROS and other free radicals (Recknagel *et al.*, 1989). However, when a condition of oxidative stress establishes, the defense capacities against ROS becomes insufficient (Halliwell and Gutteridge, 2000). ROS also affects the antioxidant defense mechanisms, by reducing the intracellular concentration of GSH and decreases the activity of SOD, GSH-Px and CAT. It has also been observed to decrease the detoxification system produced by GST (Yamamoto and Yamashita, 1999).

It is a known fact that MEL, the chief secretory product of the pineal gland, was found to be a multi-faceted free radical scavenger and antioxidant. It detoxifies a variety of free radicals and reactive oxygen intermediates, including the hydroxyl radical, singlet oxygen peroxy nitrite anion and nitric oxide (Tan *et al.*, 2002). In both *in vitro* and *in vivo* experiments, MEL has been found to protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes, such as the  $CCl_4$ , ischemia reperfusion, amyloid-protein, carcinogen safrole and ionizing radiation (Marchiafava and Longoni, 1999). Melatonin also has been reported to stimulate the activities of enzymes and increase gene expression that improves the total antioxidative defense capacity of the organism such as SOD, GSH-Px, and GRH (Kotler *et al.*, 1998). Recent studies indicate that MEL is effective on inhibiting oxidative liver damage (Calvo *et al.*, 2001). Melatonin also could dose-dependently reduce liver lipid peroxide content in  $CCl_4$ -treated rats. This indicated that MEL exerted a therapeutic effect on  $CCl_4$ -induced acute or chronic liver injury in rats, possibly through its antioxidant action. Melatonin plays a cytoprotective role in the liver insulted by ischemia and reperfusion by virtue of its ability to prevent hepatic malfunction and inhibit the generation of free radicals and accumulation of neutrophils in the damaged hepatic tissue (Lepay *et al.*, 1985). The present study showed that MEL decreased MDA content in liver homogenates and SOD activity rose significantly.

It was reported that changes in intracellular cation homeostasis are closely related to the mechanism of hepatic cell injury (Gasbarrini *et al.*, 1992; Carini *et al.*, 1998). Elevation of  $Ca^{2+}$  concentration is associated with the development of cell damage (Orrenius *et al.*, 1992). In the present study,  $CCl_4$  caused increase in both serum and subcellular fractions of  $Ca^{2+}$ , suggesting that this dramatic elevation is closely related to  $CCl_4$ -induced hepatocellular injury. However,  $Ca^{2+}$  level elevation in rats with liver injury after MEL treatment was significantly decreased than that in  $CCl_4$ -treated groups, indicating that MEL prevented disturbance of  $Ca^{2+}$  homeostasis. It has also been observed that subcellular  $Ca^{2+}$  elevation is caused by the decreased activities of  $Na^+/K^+$ - and  $Ca^{2+}$ -ATPases in hepatocyte membranes (Srivastava *et al.*, 1990). In addition, it is possible that  $CCl_4$  cause the release of  $Ca^{2+}$  by inhibiting the endoplasmic reticulum  $Ca^{2+}$

pump so that a passive leak of  $\text{Ca}^{2+}$  from endoplasmic reticulum resulted in the increase in  $\text{Ca}^{2+}$  and induces cell death (Cheng and Jan, 2002).

It has been established that TNF- $\alpha$  is a multifunctional cytokine, mostly secreted by inflammatory cells and is involved in numerous pathological states (Muto *et al.*, 1988). TNF- $\alpha$  is considered to be a common early effector molecule for liver injury, in addition to its direct cytotoxic effects, this cytokine is able to induce chemokines, macrophage chemotactic protein-1, adhesive molecules and vascular-cell adhesion molecule-1, which are keys to inflammation and consequent liver damage (Arthur *et al.*, 1985). Prevention of liver injury has been observed upon neutralization of TNF- $\alpha$  with anti-TNF- $\alpha$  antibody. Also, prevention of translation of primary RNA transcript of TNF- $\alpha$  by antisense oligonucleotide and interaction of TNF- $\alpha$  with soluble TNF- $\alpha$  receptors protects the liver (Van Zee *et al.*, 1992). Although, IL-1 itself does not exert damage on liver, its elevation could stimulate inflammatory cells to secrete many other cytokines including TNF- $\alpha$ , IL-6 and IL-8. Our findings suggest that the elevation of inflammatory cytokines including TNF- $\alpha$  and IL-1 in serum contributes to the mechanisms of inflammatory liver injury. Thus suppression of TNF- $\alpha$  and IL-1 could be one of the means by which MEL attenuated inflammatory liver injury in rats. Ogeturk *et al.* (2004) found that administration of MEL before or simultaneously with  $\text{CCl}_4$  decreased serum TNF- $\alpha$  levels and improved survival on rats.

Histopathologically, changes indicative of liver injury were more pronounced and found more frequently in  $\text{CCl}_4$ -treated rats compared with the control rats. Also the scores for hepatocellular injury were significantly higher in  $\text{CCl}_4$ -treated group of rats. There were varying degrees of inflammation, necrosis, vacuolation and vascular changes which were more pronounced in  $\text{CCl}_4$ -treated rats than control and MEL-treated groups of rat. Other histopathological changes which are unique with  $\text{CCl}_4$ -treated rats were increased amounts of endoplasmic reticulum, leading to swollen hepatocytes and ground-glass appearance of the cytoplasm. These findings are in agreement with the observations of Gaskill *et al.* (2005). At the same time,  $\text{CCl}_4$  has been reported by others to produce a rapid and prolonged depletion of liver glycogen in rats (Hickenbottom and Hornbrook, 1971). In this study,  $\text{CCl}_4$ -treated rats had hepatic glycogen contents lower than those of control group of rats. These changes in hepatic glycogen metabolism produced by  $\text{CCl}_4$  might be associated with a decreased activity of the I-form of glycogen transferase and an increased activity of glycogen phosphorylase. The effects of  $\text{CCl}_4$  treatment on hepatic glycogen metabolism were not due solely to structural damage of parenchymal cells since they were still found when the  $\text{CCl}_4$ -induced morphological changes were substantially prevented by pretreatment with melatonin. The changes in the activity of glycogen transferase and phosphorylase produced by  $\text{CCl}_4$  treatment were related to a decreased activity of glycogen transferase phosphatase and phosphorylase phosphatase enzymes. These changes might be caused in part by the ability of  $\text{CCl}_4$  to inhibit or decrease hepatic protein synthesis.

In conclusion, melatonin is protective against oxidative liver damage and liver fibrosis induced by  $\text{CCl}_4$ . Therefore, the ameliorative effects of melatonin on inflammatory and fibrotic liver injury might be related to free radical scavenging action and antioxidant activity by, increased SOD content, and decreased expression of pro-cytokines.

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